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Critical Review

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Meta-analysis of fish early life stage tests

Meta-Analysis of Fish Early Life Stage Tests – Association of Toxic Ratios and Acute-To-Chronic Ratios with Modes of Action

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Abstract: Fish early life stage (FELS) tests (OECD test guideline 210) are widely conducted to estimate chronic fish toxicity. In these tests, fish are exposed from the embryonic to the juvenile life stage. In order to analyse whether certain modes of action are related to high toxic ratios (TR, i.e., ratios between experimental effect and baseline toxicity) and/or acute-to-chronic ratios (ACR) in the FELS test effect concentrations for 183 compounds were extracted from the US EPA ecotoxicity database. Analysis of effect concentration of narcotic compounds indicated that baseline toxicity could be observed in the FELS test at similar concentrations as in acute fish toxicity test. All non-narcotic modes of action were associated with higher TRs with median values ranging from 4 to 9.3×10^4 (uncoupling < reactivity < neuromuscular toxicity < methemoglobin formation < endocrine disruption < extracellular matrix formation inhibition). Four modes of action (were also found to be associated with high ACRs: (1) lysyl oxidase inhibition leading to notochord distortion, (2) putative methemoglobin formation or haemolytic anemia, (3) endocrine disruption, and (4) compounds with neuromuscular toxicity. It was discussed that for the prediction of effect concentrations in the FELS test with alternative test systems, endpoints targeted to the modes of action of compounds with enhanced TR or ACR could be used to trigger FELS tests or even replace these tests. This article is protected by copyright. All rights reserved

Keywords: Fish early life stage test, Adverse outcome pathways, Baseline toxicity, Mode of action, Alternatives to animal testing, Fish embryo test

INTRODUCTION

The assessment of chronic fish toxicity is an integral part of environmental risk assessment for the registration of industrial chemicals, pesticides, biocides and pharmaceuticals around the globe (Scholz et al., 2013b). An early analysis of fish life cycle tests indicated that in most cases the embryo-larval and juvenile stages were the most sensitive in response to chemical exposure (McKim, 1977). Therefore, the fish early life stage (FELS) test has been used ever since as a lower tier test to detect chronic fish toxicity (Oris et al., 2012; Scholz et al., 2013b). In contrast, full life cycle or multi-generation tests are only conducted in rare cases and for higher tier testing. An appropriate OECD test guideline (OECD TG 210) is available for regulatory testing and endpoints measured include hatching success, pre- and post-hatch survival, growth (indicated by length and weight at the end of the test), and developmental abnormalities. The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) are typically reported for each endpoint (Oris et al., 2012).

Given that the FELS test requires the highest number of vertebrates in environmental hazard assessment, and exposed animals may suffer from pain or distress, various approaches have been proposed to replace this test (Scholz et al., 2013b). For instance, Volz et al. (2011) described a three-tiered testing strategy using cellular assays as an initial (tier 1), embryo testing as intermediate (tier 2), and FELS test as a confirmatory test (tier 3). In a subsequent publication it was suggested to identify mechanisms leading to FELS toxicity and develop an optimised fish embryo test (Villeneuve et al., 2014). Both suggestions relied on the use of the adverse outcome pathways (AOP) concept, i.e. the assumption that any adverse effect (AO) of a chemical on an individual or a population is propagated from a molecular initiating event (MIE), through perturbations of key events (KE) at the cellular, tissue and organ levels (OECD, 2012; Ankley et

al., 2010; Kramer et al., 2011; Ellison et al., 2011). Both Volz et al. (2011) and Villeneuve et al. (2014) suggested various potential MIEs and KEs as central to the development of FELS toxicity such as arylhydrocarbon receptor (AhR) binding, acetylcholinesterase (AChE) inhibition, gill cell toxicity and swim bladder inflation. For the swim bladder inflation, a potential link of FELS toxicity to inhibition of the enzyme thyroid peroxidase and subsequent reduction of thyroid hormone levels was experimentally demonstrated by exposure of fathead minnow and zebrafish to 2-mercaptobenzothiazole (Nelson et al., 2016; Stinckens et al., 2016). However, a detailed analysis of existing FELS data aiming to identify major AOPs associated with chronic toxicity is lacking.

In order to identify relevant AOPs, FELS toxicity could be reviewed from two different angles: the identification of compounds with high toxic ratios (TR) or high acute-to-chronic ratios (ACRs). The TR represents the relationship of the experimental toxicity effect concentration (EC) versus the EC for baseline toxicity calculated with a Quantitative Structure Activity Relationship (QSAR). Baseline toxicity or narcosis is representing the unspecific interaction of chemicals, resulting in constant internal membrane concentrations that cause acute toxicity in aquatic organisms (reviewed in e.g. van Wezel and Opperhuizen, 1995). Since internal concentrations in aquatic organisms are driven to a large extent by partitioning of the chemical between water and the organism, the baseline toxicity is related to descriptors of the hydrophobicity of a compound, e.g. the partition coefficients for octanol and water (K_{ow}) or lipid and water (K_{lipw}). Differences in the toxicokinetics of a compound may, however, interfere with the internal concentrations. The concept of baseline toxicity has been widely used for the identification of specific modes of action that cause acute toxicity at a considerably lower internal concentration (Escher and Hermens, 2002). It has been applied to derive structural alerts

or modes of action impacting on acute toxicity of various organisms (e.g. Russom et al., 1997; Verhaar et al., 1992; Maeder et al., 2004; Von der Ohe et al., 2005). In order to derive TRs, typically a regression analysis of LC_{50} and the log K_{ow} for compounds with a known narcotic mode of action is conducted to derive baseline toxicity levels. In contrast to acute toxicity, baseline toxicity regression of the FELS test has been only analysed for a limited number of structurally related narcotic compounds (chlorobenzenes, Van Leeuwen et al., 1990b). Alternatively, specific modes of action may be identified by application of the constant toxic membrane concentration (CTMC, Escher and Hermens, 2002) or the chemical activity concept (Schmidt and Mayer, 2015). The latter has already been applied to FELS toxicity for a limited number of hydrocarbons (Butler et al., 2016) and for a 14-d exposure of 50 industrial chemicals in guppy (Mayer and Reichenberg, 2006).

The ACR is defined as the quotient of effect concentrations for acute toxicity and chronic toxicity, typically the quotient of LC_{50} and NOEC. An ACR close to 1 would indicate compounds for which a similar range of effect concentrations would be found in acute and chronic exposure scenarios. The toxicity of compounds with a high ACR would not be sufficiently described by acute toxicity tests. ACRs have often been calculated to provide assessment factors to extrapolate from acute to chronic toxicity (e.g. Forbes and Calow, 2002; Slooff et al., 1986; Elmegaard and Jagers op Akkerhuis, 2000; ECETOC, 2003). A greater value of ACRs might, however, be seen in the possibility to provide information on compound characteristics and mechanisms leading to chronic toxicity. For instance, a study by Kenaga (1982) revealed particular high ACRs for insecticides, herbicides and benzene substitutes in fish and invertebrates. Ahlers et al. (2006) detected a tendency of narcotic compounds (i.e., baseline toxicants) to show low ACRs. Various structural alerts indicating a higher probability to provoke

increased chronic toxicity, i.e. high ACRs, were identified (Ahlers et al., 2006). A study of Roex et al. (2000) showed that non-polar narcotics exhibited the smallest variation in ACRs. It was concluded that for this group of compounds chronic effects could be predicted using acute toxicity tests. A mechanistic explanation would be that narcosis occurs at a constant membrane concentration or membrane volume fraction (Warne et al., 1991), independent or only slightly dependent on the exposure duration (Chaisuksant et al., 1997), and is reversible, i.e., disappears completely once the chemicals are depurated (van Wezel and Opperhuizen, 1995).

Higher ACRs were observed particularly for compounds with specific modes of action (MoAs) (herbicides, central nervous system seizure agents and acetyl cholinesterase inhibitors) in fish and invertebrates (ECETOC, 2003). Both, the study of Kenaga (1982) and ECETOC (2003), suggested that also modes of action for which the toxicity could be expected to be established at the acute exposure levels may exhibit high ACRs. However, a systematic evaluation whether a potential cumulative damage, increasing susceptibility, toxicokinetics and/or potential differences in the mode of action lead to high ACRs is lacking.

The main aim of our study was to conduct a meta-analysis on the FELS test in order to identify potential MoAs that could be used to design MoA- and AOP-targeted assays. From many available analyses of chronic fish toxicity data, however, it is difficult to derive indicators for modes or mechanisms of actions that lead to high TRs or ACRs in the FELS test, since they had different goals. In many of these studies, the identity of the compounds has not been revealed in the publication, data from different species, animal classes (e.g. invertebrates and fish) or different type of tests (e.g. full life-cycle, reproduction, early life stage) were combined, the analysis was limited to a low number of compounds and/or the evaluations were focused on experimental design and statistics (Ahlers et al., 2006; ECETOC, 2003; Elmegaard and Jagers op

Akkerhuis, 2000; Roex et al., 2000; Suter and Rosen, 1988; Slooff et al., 1986; Oris et al., 2012; Van Leeuwen et al., 1990b). In comparison to earlier analyses (e.g. Kenaga, 1982), the database was extended and included a wider and more systematic approach (regression-based ACRs, baseline toxicity analysis). Given that, principally an unlimited number of organic compounds could be expected for future chemical development, our analysis was restricted to organic compounds. FELS effect concentrations from publically available studies were compared to corresponding acute fish and fish embryo LC₅₀ data. A particular focus was given on the embryonic stages on the FELS test and the fish embryo test, since the embryonic stage may provide a key to predict FELS toxicity and to develop alternative test systems.

MATERIALS AND METHODS

Data base search

We searched the US-EPA ECOTOX database for FELS toxicity studies (<http://cfpub.epa.gov/ecotox>) with organic compounds reported until December 2015. This was done by an initial search for fish studies that reported NOEC and/or LOEC values (which are typically reported for FELS tests) for a minimum of a 32-day exposure in warm water species and 60-day for rainbow trout. The search was limited to studies using one of the seven major species for which FELS tests are mainly conducted, i.e. fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), sheepshead minnow (*Cyprinodon variegatus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), flagfish (*Jordanella floridae*) and mummichog (*Fundulus heteroclitus*). These species are commonly used in ecotoxicity. Nevertheless, for some of them, such as mummichog, only 4 studies/compounds with FELS tests were identified. Therefore, to avoid a too heterogeneous data set with respect to species, all other species than those indicated above were not considered in the data search. Subsequently, studies that referred

to FELS tests conducted similar as described in the OECD 210 guideline were manually selected. As a minimal criterion, it was required that exposure was initiated within 24 or 48 (fathead minnow) hours post fertilisation (hpf). Additionally, we searched the open literature (ISI web of science, google scholar) for FELS studies. FELS studies that indicated purities of the test compound below 90% were excluded. Wherever possible (i.e., in all cases where the data stemmed from a published journal article or publically available report), the original study was used to extract the effect concentration (indicated in supplement table S1). In cases where original data were not publically available (22 %), the US-EPA database entry was used. For correlation analysis only studies were considered that reported both a NOEC and a LOEC for either survival or growth (length and weight of fish at the end of the test).

LOECs for growth (length and weight at the end of the test) exhibited a strong correlation with survival LOECs, occurring on average at 2-3fold lower concentrations. Therefore, in cases where no survival rates (LOEC/NOEC pairs) were available, these were predicted from the LOEC for growth or vice versa based on the regression equations (Fig. S5). This was done in order to combine studies for which LOEC/NOEC pairs were only available for either survival or growth and to increase the number of data points for the subsequent comparison with acute fish or fish embryo toxicity data. However, it must be noted that a few compounds such as 17 α -methyltestosterone exhibited a significantly higher sensitivity for growth and hence, effect concentrations for survival rates might be underestimated in a few cases.

After retrieval of FELS toxicity data, corresponding acute 96-h fish toxicity data were identified for the same species that was used in the FELS study. For fathead minnow, most acute fish toxicity data were obtained from the Duluth database (Russom et al., 1997). The acute toxicity data of other species were retrieved from the US-EPA ECOTOX database.

Corresponding zebrafish embryo LC₅₀ stemming from studies using diverse protocols and variable exposure times were identified from a previously established database (Scholz et al., 2014; Scholz et al., 2016). In case of multiple studies conducted for one compound and species, the geometric mean of the LC₅₀ was used. Physico-chemical properties (molecular weight, log K_{ow} , water solubility) were calculated from SMILES codes using EPISUITE v4.11 (<https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface>) and ACD Labs v12.5 (Build 39480). All values from the EPISUITE v4.11 program were assumed to represent the neutral form of the chemical. The degree of ionization was calculated by treating all chemicals as monoprotic acids or bases and using the most influential p K_a (predicted by ACD Labs) to calculate the degree of ionisation at pH 7. Compounds with a degree of >50 % ionisation were labelled and excluded from the regression analysis for baseline toxicity (narcotics). The 50 % ionization level was chosen to highlight compounds, for which the ionization may impact on uptake and - if not considered - lead to an underestimation of baseline LC₅₀ and TRs. Ionization could principally be considered by replacing the log K_{ow} with the log K_{lipw} (membrane water partition coefficient) and calculating the K_{lipw} for the charged species. However, for neutral compounds the K_{ow} is very similar to the K_{lipw} (Endo et al., 2011) and the partitioning of charged species is often within a factor of approximately 10 compared to the corresponding neutral species for most classes of IOCs (Bittermann et al., 2016). Hence, the impact on baseline toxicity and/or TR calculation of ionizable compounds is limited and may only be observed with higher degrees of ionization.

Correlation analysis

Regression analysis of molar effect concentrations was conducted using a Deming (type II) regression in order to consider variability for both the independent and dependent variable.

The regression analysis was performed using the Software Sigma Plot 12.0 (Systat Software GmbH, Erkrath, Germany) or the R-package mrc (R Core Team, 2015). Statistically significant deviation of the regression slope from 1 or -1 was calculated with the F-test in Sigma Plot 12.0 ($p < 0.05$). Compounds deviating from the regressions were identified with a box plot analysis of the regression residuals using the software IBM SPSS Statistics version 21 (IBM, Ehningen, Germany) based on a deviation from the regression by more than 1.5 fold of the interquartile distance below or above the 25 or 75 % percentile. After the box plot analysis the regression analysis was repeated excluding the previously identified deviating values in order to avoid a distortion of the regression by individual values deviating from the regression.

Calculation of toxic ratios and acute chronic ratios

LC₅₀s are typically obtained from modelled concentration-response curves to describe acute toxicity. They are used for describing the relationship between physico-chemical and structural characteristics for acute aquatic toxicity and to derive TRs from the comparison of predicted LC₅₀ for narcotic compounds versus observed LC₅₀s (e.g. Maeder et al., 2004). In contrast, NOEC and LOEC values are used to describe chronic fish toxicity based on concentrations where a statistically significant difference in the effects (e.g. survival, growth) is observed. The disadvantage of NOEC and LOEC is their dependency on the sample size and variability of the effect. Hence, ideally modelled effect concentrations would also be used to analyse FELS toxicity. Unfortunately, for many of the publically available chronic toxicity studies the raw data are not provided and it is difficult to reanalyse data for modelling of effect concentration. However, if NOEC and/or LOEC data would correlate to modelled effect concentrations they could be used as a surrogate. In order to demonstrate the relationship of the NOEC/LOEC to the LC₁₀ and LC₅₀ for survival we analysed the data of one study with a larger

compound set including compounds with different MoAs and availability of all pertinent raw data (Call and Geiger, 1992). Concentration-response curves for survival in the FELS test were fitted to the data using the Hill-slope equation (eq. 1) and used to estimate LC₁₀ and LC₅₀ values.

$$y = Min + \frac{Max - Min}{1 + (\frac{x}{LC_{50}})^{-p}} \quad (1)$$

The parameter Max was set to 100 % and the slope (p) was estimated. Given relatively high background mortality in some replicates of the compound tested, the parameter Min was estimated as well. This means that LC₁₀ or LC₅₀ may in some cases not represent 10 or 50 % lethality levels, respectively, but different percentages of mortality. However, this approach was required as 10 % effect levels would otherwise be difficult to calculate, in part, due to the limited number of compounds (n=24) for which concentration-dependent mortality data were available. The independent variable *x* represents the measured exposure concentration [μM] and *y* the percentage of survival [%]. The software R and the package drc (R Core Team, 2015) embedded into a KNIME workflow were used to model concentration-response curves (Berthold et al., 2008) (see supplement Fig. S3 for concentration response modelling).

A strong correlation (Pearson's correlation coefficient = 0.99) and similar sensitivity (slope between 0.97 and 0.99 and intercept of -0.09 or 0.01 respectively) were found for both the comparison of log transformed NOEC_{survival} to the LC₁₀ and LOEC_{survival} to the LC₅₀ (supplement Fig. S4). Regression analysis indicated a slope not significantly different from 1 for both comparisons and the regression lines were very close to the line of unity. Maximum differences of 4.7 (LC₁₀ versus NOEC) and 2.8 (LC₅₀ versus LOEC) were observed. Given the high correlation of LOECs and LC₅₀s we used LOECs for the calculation of TRs (eq. 2) with the

LOEC_{baseline toxicity} representing the predicted baseline toxicity LOEC for narcotic compounds and the LOEC_{exp} representing the statistically significant different LOEC from an experimental study. Baseline toxicity is believed to reflect the nonspecific disturbance of the integrity and functioning of cell membranes by chemicals (Escher et al., 2011). At a log $K_{ow} < 0$, the internal cellular bioavailable concentrations will equal the external exposure concentration and hence, membrane accumulation will be relatively low and potentially less important for unspecific toxicity. Hence, in order to avoid overestimation of TRs, a minimum log K_{ow} of 1 was used for baseline toxicity and TR calculation.

$$TR_{FELST} = \frac{LOEC_{baseline\ toxicity}}{LOEC_{exp}} \quad (2)$$

Data derived from the marine species *C. variegatus* and *F. heteroclitus* were not corrected for the impact of ionic strength on solubility and partitioning, which might impact on effect concentrations for baseline toxicity and possibly also other types of toxicity. As indicated in previous publications (Gouliarmou et al., 2012; Escher et al., 2017) the salting out effect on partitioning and solubility are of limited significance at least when assessing LOEC values covering more than eight orders of magnitude and presenting these values in log-log plots.

The ACR represents the ratio of acute versus chronic toxicity. There is no agreement in the literature with regard to the effect concentrations to be used for the calculation of ACR but mostly the LC₅₀ for acute toxicity is compared to the NOEC of chronic toxicity (e.g. Ahlers et al., 2006). Given the strong correlation of LC₅₀s and LOECs for the FELS test we have calculated the ACR based on the LOECs for survival. We derived three different ACRs depending on the acute toxicity data that were used as reference (eq. 3-5).

$$ACR_{int} = \frac{LOEC_{embryo\ toxicity}}{LOEC_{FELST, survival}} \quad (3)$$

$$ACR_{AFT} = \frac{LC_{50, AFT}}{LOEC_{FELST, survival}} \quad (4)$$

$$ACR_{ZFET} = \frac{LC_{50, ZFET}}{LOEC_{FELST, survival}} \quad (5)$$

The ACR_{int} (int=intrinsic) describes the ratio of the observed toxicity in the embryonic phase of the FELS test ($LOEC_{embryo\ toxicity}$) to the LOEC of the entire FELS test ($LOEC_{FELST, survival}$, eq. 3). Some studies do not distinguish between teratogenic effects and mortality and hence, the $LOEC_{embryo\ toxicity}$ may in some cases include sublethal effects as well. A high ACR_{int} would indicate that the mortality would increase after the embryonic phase. Given the lack of LC_{50} data for embryo toxicity in FELS tests, the LOEC was used. The ACR_{AFT} is based on the comparison of acute fish 96-h toxicity LC_{50} s ($LC_{50, AFT}$) and the $LOEC_{FELST, survival}$ of the same species. This type of ACR, i.e., comparison to the acute toxicity of juvenile or adult fish is most commonly used for deriving ACRs. LC_{50} s and LOECs for the $LOEC_{FELST, survival}$ may be derived from different studies. The ACR_{ZFET} compares acute zebrafish embryo toxicities ($LC_{50, ZFET}$) with the $LOEC_{FELST, survival}$ test.

Calculation of reference lines for effective chemical activity and the CTMC

In order to estimate whether baseline toxicity in the FELS is within the range of baseline toxicity typically observed for narcotic compounds in acute toxicity, reference lines for effective chemical activities (a) of 0.01 and 0.1 and a CTMC of 100 mM were calculated. For the

calculation of effective chemical activities we used the generalised solubility equation of Ran and Yalkowsky (Ran and Yalkowsky, 2001) to derive the water solubility of the corresponding subcooled liquids (S_w^{liquid} , eq. 6) and to calculate the corresponding effect concentration for a given $\log K_{ow}$ (eq. 7).

$$\log S_w^{\text{liquid}} (\text{mol/L}) = 0.5 - \log K_{ow} \quad (\text{eq. 6})$$

$$a = \log \frac{\text{effect concentration } [\frac{\text{mmol}}{\text{L}}]}{S_w^{\text{liquid}} [\frac{\text{mmol}}{\text{L}}]} \quad (\text{eq. 7})$$

The CTMC reference line (eq. 8) for exposure concentrations causing narcosis ($C_{\text{water,narcosis}}$) at equilibrium conditions (Goss and Endo, 2016) was calculated using the K_{ow} instead of the membrane partition coefficient given the strong concordance of both partition coefficients for neutral compounds (Endo et al., 2011).

$$C_{\text{water, narcosis}} = \frac{100 \text{ mmol/L}}{K_{ow}} \quad (\text{eq. 8})$$

Identification of modes of action

The MoAs of all compounds included in the analysis were identified primarily related to the intentional use and by considering whether the appropriate target would be present in fish. For instance, an acetylcholinesterase inhibiting insecticide would target the synapse of cholinergic nerve cells which are abundant in fish, albeit it may be less important due to metabolic degradation or weaker receptor/enzyme affinity. In contrast, a photosystem II

inhibition caused by a herbicide represents the primary intended mode of action but this MoA is not relevant for heterotrophic organisms. Hence, in case that no primary mode of action was available for fish or at least vertebrates, publically available literature or reports from governmental agencies were searched in order to deduce a presumable MoA. In case that no data on the primary or presumable MoA were available, they were predicted for acute toxicity based on a modified structural alert approach (Ellison et al., 2015) of Verhaar et al. (1992) using the software Toxtree 2.6.13. Oxidative phosphorylation uncouplers were identified using a combination of structural alerts based on Russom et al (Russom et al., 1997; Schultz and Cronin, 1997) conducted with the software ChemProp (UFZ, 2016), and/or experimental data summarised by Escher et al. 2002 (Escher and Schwarzenbach, 2002). Experimental evidence provided by Escher et al. 2002 (Escher and Schwarzenbach, 2002) was preferred in case of contradictory classifications. Furthermore, the classifications were checked for agreement with structural requirements for uncoupling (Terada, 1990).

RESULTS

Establishment of an FELS test data collection

Effect concentrations (NOEC and/or LOEC) were identified for 183 compounds from the US-EPA ECOTOX database and the open literature (a detailed table with CAS-RN, chemical properties, effect concentration, modes of action and references for each compound is given in Table S1 and S2 of the supplement information). These data represent 258 database entries, since some compounds have been studied in more than one species or study. FELS data were found for 131 (*Pimephales promelas*), 50 (*Oncorhynchus mykiss*), 22 (*Cyprinodon variegatus*), 24 (*Danio rerio*), 17 (*Oryzias latipes*), 10 (*Jordanella floridae*) and four compounds (*Fundulus heteroclitus*). Additional 41 compounds/studies were not included in subsequent analyses due to

a test compound purity of <90 % or because of obvious deviations from the OECD 210 guideline (exposure was started after 48 hpf or after hatching). For 14% of the FELS data entries with available NOEC/LOEC pairs, nominal exposure concentrations were not verified by chemical analysis. Information on the purity of the test chemical was lacking for 27% of the data entries. The comparative analyses were limited to compounds and studies respectively that derived both a LOEC and a NOEC (164 compounds and 231 study entries, respectively).

Compounds with high toxic ratios in the FELS test

Using the dataset established for this study, it was possible to establish a baseline toxicity regression and to calculate the corresponding toxic ratios for a wider set of compounds. The baseline toxicity regression was obtained by comparing the $LOEC_{FELST}$ of all neutral narcotic compounds with the corresponding $\log K_{ow}$. A narcotic mode of action was assigned to all neutral compounds that were not known or not hypothesized to exhibit a specific or reactive MoA and/or were predicted as acute non-polar narcotic chemicals using structural alerts defined by Russom et al.(1997) and Verhaar et al. (1992). The LOEC was used for this analysis given the high concordance with the LC_{50} (see *Calculation of toxic ratios and acute chronic ratios*). The regression analysis revealed a slope not significant different from -1 and an intercept of 1.73 (Fig. 1).

We then used the baseline regression to calculate the TR. Compounds that could not be associated with a MoA or with a presumed uncoupling mode of action in acute toxicity tests exhibited the lowest TR with median values ≤ 4.3 (Fig. 2). Increasing TRs were observed for neurotoxic compounds, compounds potentially provoking methemoglobin formation, potential endocrine disrupters and compounds with other specific MoAs (e.g. mutagenic compounds, alcohol dehydrogenase inhibitors). The highest TR ranges were obtained for dithiocarbamate

fungicides interfering with extracellular matrix formation as potential MoA (TRs ranging from 7.2 to 3×10^9). However, in all groups compounds with a low TR were found as well. TRs well below one indicate that exposure concentrations may have been underestimated due to experimental limitations or a reduced internal bioavailability.

The observed FELS test effect concentration were compared to chemical activities of 0.1 and 0.01 and a CTMC of $100 \text{ mmol/kg}_{lip}$, which have been both shown to characterise the acute baseline toxicity (narcosis) of neutral organic compounds. FELS toxicity data were found around chemical activities of 0.01 for narcotic compounds and were close to the reference line of a CTMC of 100 mM (Fig. 1).

ACR: Comparison of acute fish and FELS toxicity

The TR analysis of the FELS test revealed diverse MoAs with effect concentrations well below the baseline toxicity. Several of the described MoAs (uncoupling, reactivity, neurotoxicity) had been identified in previous analyses (e.g. Verhaar et al., 1992; Russom et al., 1997) with high toxic ratios for acute toxicity. Hence, the low ACR of these compounds may indicate that FELS toxicity is already established during an acute exposure. We compared three different types of ACR: The LOEC for embryonic phase of the FELS test ($LOEC_{FELST}$, embryo toxicity), the LC_{50} for acute 96-h fish toxicity ($LC_{50, AFT}$) and the acute zebrafish embryo toxicity ($LC_{50, ZFET}$). These reference values were used to calculate corresponding ACRs (ACR_{int} , ACR_{AFT} , ACR_{ZFET}) with equations 3 to 5.

ACR_{int}

FELS tests include the exposure of embryonic stages and the toxicity of many test compounds might already be fully established during acute exposure in the embryonic period. A low ACR_{int} would indicate that continuation of exposure beyond the embryonic stage would not

result in an increased toxicity. Furthermore, the ACR_{int} was derived from the same experiment and hence is not vulnerable to experimental variability between different studies. Therefore, we compared the LOECs for embryo toxicity (cumulative teratogenicity and survival) with the LOECs obtained for overall survival and growth of the entire test duration. A high concordance was observed for 36 data pairs for which LOECs for embryo toxicity and the entire test duration were available (Fig. 3A). The regression slope of 0.99 and intercept of 0.077 (for comparison of logarithmic values) was close to the line of unity and indicated nearly equal sensitivity of survival LOECs for the embryonic and entire test period. The slightly higher sensitivity for some compounds in the embryonic phase results from the cumulative assessment of teratogenicity and mortality, since separate LOECs for these endpoints were not available in some studies (Van Leeuwen et al., 1986; Van Leeuwen et al., 1990a). However, no effect on survival was observed for 31 compounds in the tested concentration range during embryonic development. For most of these compounds (29) the maximum test concentration was close to the LOEC for overall survival or growth. Hence, in order to estimate whether these compounds exhibit a considerably increased ACR higher test concentrations would need to be considered for the embryonic period. For only three compounds (with different MoA) – triethylamine, 2,4-dichlorophenol and 17-methyltestosterone – the tested range of concentrations indicated that chronic effects in the FELS test were at least tenfold below the concentrations that caused mortality in the embryonic period (Supplement Table S6).

ACR_{AFT}

The analysis of the ACR_{AFT} was based on a comparison of effect concentration for the same species. It was hypothesized that for the majority of compounds the effect concentration in both tests would be similar. Only for some compounds, presumably those with a specific MoA, a

high ACR_{AFT} might be observed. In contrast to the comparison of survival rates of the embryonic period and the overall test (see *ACR: Comparison of acute fish and FELS toxicity*) the effect concentrations of this comparison stem from different tests or studies introducing additional variability. In case that for the FELS test only a pair of LOEC/NOEC for growth was available, the corresponding effect concentrations for survival were estimated using a linear regression equation (Supplement, Fig. S5).

For the correlation analysis, 182 data pairs (LOEC / LC_{50}) referring to 128 different compounds were identified. Of these compounds 81 were tested in fathead minnow (*P. promelas*), 28 in rainbow trout (*O. mykiss*), 16 in sheepshead minnow (*C. variegatus*), 8 in flagfish (*J. floridae*), 10 in zebrafish (*D. rerio*), 8 in medaka (*O. latipes*) and/or one in mummichog (*F. heteroclitus*) (see supplement Tables S1 and S2 for the complete data set including physicochemical characteristics of the test compounds and references to original studies).

The regression analysis of logarithmic values indicated a high correlation of acute fish toxicity and FELST data with a data correlation coefficient (R) of 0.95 (Fig. 3B). The slope of the regression was not significantly different to one and the intercepts indicate a similar overall sensitivity (2.5 fold higher sensitivity for survival in the FELS test). Twelve compounds (represented by 13 studies, (Fig. 3B), were deviating for the acute fish toxicity – FELST survival regression. The ACRs of these compounds ranged from 45 to 8318 (Supplement Table S6). Five compounds did not provoke any acute toxicity in the tested range of concentrations. For these compounds ACRs greater 1.7 – 2570 can be expected based on the comparison of the FELS test with the maximum tested concentration in the acute fish toxicity test (supplement Table S6). Four compounds (trichloroethylene, neodol, aldicarb, permethrin) with higher toxicity in the

acute fish toxicity test were detected. The higher sensitivity is likely to represent an artefact or experimental variability, respectively, given that data stem from different experiments and studies.

ACR_{ZFET}

Zebrafish embryo acute is known to exhibit on average a high correlation to the acute fish toxicity with a similar sensitivity (Lammer et al., 2009; Belanger et al., 2013; Knöbel et al., 2012; Klüver et al., 2015). Hence, likewise as found for comparison of acute fish toxicity and FELS toxicity a high correlation would be expected, if zebrafish acute embryo LC₅₀ were compared to FELS LOECs (Fig. 3C). For 57 compounds of the FELS test database corresponding zebrafish embryo LC₅₀ values were available (obtained from a previously established database, Scholz et al., 2016). A regression analysis (based on logarithmic values) indicated a weaker correlation ($R = 0.52$) compared to the comparison of embryo toxicity in the FELS or acute fish toxicity. Apparently, the data were more scattered and a higher number of compounds with lower toxicity in the fish embryo was observed. Due to the more scattered data it was not possible to identify compounds with a lower effect concentration in the FELS test by a statistical analysis. Therefore, we used an $ACR_{ZFET} > 45$ as a threshold to identify compounds with an increased chronic toxicity (Fig. 3C). The factor of 45 was useful to describe outliers from the regression of acute 96-h fish toxicity and FELS correlation (see above and Fig. 3). If this threshold would be applied to the comparison of zebrafish embryo LC₅₀ and FELS data 20 % of all studies would show a weaker toxicity in fish embryos (in contrast to only 6.6 % of the studies in the 96-h acute fish – FELS toxicity comparison). Fourteen compounds tested in zebrafish embryos did not provoke mortality in the tested range of concentrations. For these compounds, a minimal expected TR based on the highest concentration tested in the zebrafish embryo was

calculated (supplement Table S7). Three compounds exhibited a minimal TR above 100 (pentachlorobenzene, methomyl, ethoprop).

Relation of high ACRs with MoAs

As indicated by the comparison of AFT (adult/juvenile and ZFET, Fig. 3B) similarity of effect concentrations in AFT and FELS toxicity may not apply for all modes of action.

Therefore, the ACRs were compared also with respect to their different MoA. Most of the MoAs exhibited ACRs close to that of narcotic compounds and were ranging between 1 and 10 (Fig. 4A). Only two MoAs – methemoglobin formation and extracellular matrix formation inhibition – appeared to exhibit a substantially higher ACR range with median values between 10 and 100 and peak values of 1066 and 4786, respectively. However, a few compounds with ACRs > 10 were also observed for most of the other mode of action.

Therefore, the transduction to changes at higher organism level and adverse effects for these MoAs (lysyl oxidase inhibition and methemoglobin formation) were graphically summarised according to the AOP concept (Supplement Fig. S9). The putative AOP for lysyl oxydase inhibition leading to enhanced chronic fish toxicity has also been submitted to the AOP wiki (<https://aopwiki.org/aops/242>). Given that evidence for the link between the different levels of the AOPs is only partially available for mammals but lacking for fish or only limited amount of data supporting these links are available, these AOPs must be considered at present as putative and may require further experimental data for confirmation.

Similar to the comparison of acute fish and FELS toxicity, potential methhemoglobin formation and extracellular matrix inhibition were found to represent MoAs with the highest ACR_{ZFET} . In contrast to the comparison with acute fish toxicity, neuromuscular toxicity and endocrine disruption were additionally identified as MoAs with high ACR_{ZFET} . The analysis of

ACR_{int} versus MoA was not conducted for embryo toxicity in the FELS test given the high concordance of embryo and FELS toxicity (Fig. 3A).

For a few compounds and studies, ACRs below 1 were observed. These low ACRs could result from variability between different species and stages, e.g. due to a different degree of metabolic degradation and clearance. Furthermore, ACRs>1 may represent artefacts related to solubility of the compounds or other experimental limitations. It must be noted that the result could also be biased due to the partially low number of chemicals representing a certain MoA. elation of the acute chronic ratio to the mode of action. ACRs were calculated using acute 96-h fish toxicity LC₅₀ (A) or acute zebrafish embryo toxicity (LC₅₀, ZFET) as references. The dashed line represents an ACR of 10. For details on the compounds and data sources, refer to supplement tables S1, S2 and S6. “Inh. Extracellular matrix” refers to the inhibition of extracellular matrix formation by lysyl oxidase inhibition. Ox. = Oxidative.

No relationship of high ACRs with hydrophobicity

Compounds may not reach equilibrium of internal bioavailable concentration within short-term exposure. Indeed, the time to reach equilibrium would be particularly dependent on the hydrophobicity of the compounds and high ACRs would reflect differences in the internal bioavailable concentrations. Hence, in order to estimate whether the level of ACR may also be driven by the hydrophobicity of compounds the ACR of narcotic compounds was compared to the log K_{ow} . No dependency of the ACR on the log K_{ow} was observed for both the ACR_{AFT} and ACR_{ZFET} (Fig. S7). The comparison was restricted to narcotic compounds as for other MoAs the different reactivity or affinity to the target site may override the influence of hydrophobicity. The mean ACRs of 5 and 5.8 reflect the on average slightly lower effect concentrations obtained for the FELS test (Fig. 3 B, C).

DISCUSSION

Using a database with 183 compound FELS data were analysed with respect to whether certain mode of action may be of particular concern for chronic fish toxicity. Despite the heterogenous data sources and relaxed quality criteria it was possible to conduct regression analyses and to determine TRs and ACRs for diverse MoAs and relate chronic toxicity in the FELS test to baseline toxicity.

Association of high toxic ratios with MoAs

Determination of the toxic ratio (i.e. the relationship of observed toxicity versus calculated baseline toxicity) can provide evidence for reactivity, a specific mode of action or other interactions leading to high toxic ratios. In order to derive toxic ratios, a regression analysis for the baseline survival LOEC using the narcotic compounds of the dataset was conducted. The obtained regression was very close to a previous baseline toxicity analysis of the FELS test that was based on NOECs of a limited number of structurally related narcotic compounds in zebrafish (chlorobenzenes, $\log \text{NOEC (mM)} = -1.09 * \log K_{\text{OW}} + 1.78$) (Van Leeuwen et al., 1990b). The similarity to this data set indicates that even heterogeneous data originating from different species and studies can be combined to analyse the role of MoA for toxicity in fish. The baseline toxicity of the FELS was also very close to the baseline toxicity described for acute fish toxicity ($\log \text{LC}_{50} \text{ (mM)} = -0.94 \log K_{\text{OW}} + 1.75$; (Russom et al., 1997)) and fish embryo acute toxicity ($\log \text{LC}_{50} \text{ (mM)} = -0.99 \log K_{\text{OW}} + 2.02$; (Klüver et al., 2016)) providing evidence that a non-specific (narcosis) mode of action can be used to link hydrophobicity and effect universally across species and life stages and that narcotic compounds are generally not associated with high ACRs. The on average slightly lower effect concentrations in the FELS test could be associated with higher internal concentrations or cumulative damage. However, given the lack of

experimental internal concentration data and detailed time-resolved survival data this cannot be concluded at present.

The analysis of the MoA-specific range of TRs indicated that many of the compounds with non-narcotic MoAs exhibited an increased TR, with putative extracellular matrix inhibition and endocrine disruption as the MoA groups with the strongest deviation from the baseline.

Within the specific MoA groups also compounds with a low TR were identified. These compounds may indicate that the MoA has not been assigned correctly, the intrinsic potency is low or that a strong metabolic degradation may result in a reduced internal bioavailability confounding the analysis of exposure-concentration based TRs. It must be noted that analysis of modes of action leading to high chronic fish toxicity TRs could be also skewed due to a bias of compounds available for this study. For instance, relatively few data were available for pharmaceuticals at the time of analysis. Given their biological activities, these compounds may provide additional MoAs not represented in the database and hence, no conclusion on the potency to provoke high TRs can be made for this group of compounds at present. However, the analyses gave a first strong indication of MoAs associated with high TRs in the FELS test.

Chemical activity and constant toxic membrane concentrations

Baseline toxicity is frequently also analysed with respect to chemical activity (Schmidt and Mayer, 2015) or the critical membrane concentration leading to acute toxicity (Escher et al., 2002). In the chemical activity concept the toxicity of a chemical is expressed as the fraction of water solubility that leads to toxicity, where the water solubility refers to the liquid state of the chemical (i.e. normal solubility for liquids and sub-cooled liquid solubility for solids) (Schmidt and Mayer, 2015). This results in lethal chemical activities (La_{50}) typically between 0.1 and 0.01 for baseline toxicants and <0.01 for compounds with a reactive or specific mode of action. The

CTMC (constant toxic membrane concentrations) concept relates the baseline toxicity of a chemical to a constant membrane concentration resulting from equilibrium partitioning. For instance, acute toxicity of narcotic compounds is related to a membrane concentration (at equilibrium) of 100 mmol/kg_{lip} (Escher and Hermens, 2002). There is some controversial discussion on the relevance of the chemical activity concept (Goss and Endo, 2016; Thomas et al., 2016) but it is beyond the scope of this article to contribute to this discussion. However, both approaches are useful to compare the range of baseline toxicity of acute and chronic toxicity for neutral compounds. This comparison confirmed that the survival in the FELS test for narcotic compounds is driven mainly by their acute baseline toxicity.

Toxicity observations above the baseline toxicity range in terms of chemical activities or CTMC could indicate a reduced uptake or metabolic transformation of compounds leading to a reduced internal bioavailability. Experimental limitations (e.g. overestimated effect concentrations or water solubility) may also result in high La_{50} or CTMCs. Toxicity below the baseline toxicity range in terms of chemical activities or CTMC could indicate a specific mode of action, reactivity or other interactions leading to high toxic ratios in a chronic exposure. La_{50} that are slightly below 0.01 may also apply to some narcotic compounds, since the narcotic mode of action has been assigned based on acute toxicity QSARs. It must be noted that 28 of the compounds in our database were estimated to be in a predominantly ionised state at pH 7. For these compounds the comparison of the toxicity to the log K_{ow} , CTMC and chemical activity may not be valid as it is biased by a potentially limited uptake and different partitioning of the predominant ionised chemical (Bittermann et al., 2016). Therefore the corresponding compounds have been labelled in Fig. 1 and are indicated in the supplement data tables (S1, S6).

Association of ACRs with MoAs and hydrophobicity

While the TR analysis clearly indicates that reactivity or specific MoAs result in FELS effect concentrations well below the baseline toxicity, it may not indicate MoAs that are specifically relevant for a chronic exposure scenario or that become relevant during the development from embryo to juvenile stage. The comparison of the LOECs for embryotoxicity and overall survival in the same FELS test did not reveal any information regarding MoAs specifically relevant for chronic toxicity. This was due to the lack of LOEC data for the embryonic phase of the test, a limited exposure concentration range (46% of the compounds did not provoke toxicity in the embryonic phase) and the high correlation for the compounds for which this information was available. A high correlation and similar sensitivity was also found for the comparison of AFT and FELS test confirming previous observations (Suter and Rosen, 1988). However, the AFT-FELS test comparison indicated putative methemoglobin formation or extracellular matrix inhibition with ACRs increased by on average more than a factor of ten. Hence, all other MoAs that exhibited high TRs for the FELS test, apparently resulted in high TRs already in a short-term exposure setup. That the ACR were on average low (i.e. close to 1) is probably also determined by the similarity of baseline toxicity for AFT and FELS tests, since about 30 % of the compounds of the comparative analysis were assigned a narcotic MoA. Interestingly, additional MoAs – neuromuscular toxicity and endocrine disruption - were found to lead to high ACRs when they were calculated based on acute fish embryo toxicity. This indicates a weakness of the fish embryo with regards to the acute toxicity (lethality) that has been previously described for neurotoxic compounds (Scholz et al., 2016; Klüver et al., 2015; Knöbel et al., 2012; Glaberman et al., 2016). Some of the compounds with a high ACR_{ZFET} (aldicarb, azinphos-methyl, dieldrin, permethrin) were also identified as compounds with a weak

sensitivity in fish embryo if compared to acute fish toxicity LC₅₀ (Klüver et al., 2015) and represented acetylcholinesterase inhibitors, GABA antagonists and voltage-gated sodium channel antagonists. Furthermore, if zebrafish embryo acute toxicity and AFT data were compared, neurotoxic compounds represented the compounds with the lowest sensitivity in the fish embryo test (supplement Fig. S8).

Hydrophobicity of compounds

The internal concentration maxima may have not been reached within the experimental period of acute tests or during the embryonic stages of the FELS test. This could result in high ACRs in tests with a prolonged exposure such as the FELS test. Given that the uptake and elimination of a compound is driven to a large extent by its hydrophobicity, the relation of high ACRs to the log K_{ow} may provide information on the role of toxicokinetics for evolving chronic toxicity. Higher ACRs have been observed for log K_{ows} above 4 and associated with prolonged time to reach a steady state in bioconcentration (Ahlers et al., 2006). Higher ACRs may also be expected for polar compounds. For fish embryos it has been shown that equilibrium internal concentrations may not be approached during a 4-day exposure period for hydrophobic and also polar compounds (Brox et al., 2016; Brox et al., 2014; Klüver et al., 2015). However, the present study could not reveal any association between ACRs of narcotic compounds and hydrophobicity regardless whether the ACR was calculated based on acute fish or acute fish embryo toxicity. However, it must be noted that only a limited number of compounds and data was available and may also be confounded by unknown species-specific differences in toxicokinetics, active transport or uptake by ingestion (e.g. Luckenbach et al., 2014; Neuwoehner and Escher, 2011). A dependency on hydrophobicity may also contribute to high ACR for non-narcotic compounds but would be difficult to conclude due to the overlaying specific MoAs.

MoAs and AOPs of compounds with high TRs and ACRs

The data analysis suggests that a specific MoA increases the likelihood that a compound is provoking a high TR and/or ACR in the FELS test. This could be related partially to the manifestation of certain key events (e.g., respiratory distress) during the course of development or specific interference with growth (e.g., for endocrine disrupting chemicals or hormones).

Putative hemoglobin oxidation (associated with aniline derivatives), interference with cellular matrix formation (associated with dithiocarbamate fungicides), neurotoxicity (mainly acetylcholinesterase inhibiting organophosphates and carbamates) and endocrine disrupting or hormonally active compounds were found in a higher proportion among compounds with high TR and/or ACR (Figures 2 and 4) in the FELS test. For compounds with an aniline structure (aniline, diuron, propanil, 3,4-dichloroaniline, 4-chloroaniline, 2-imidazolidinethione), the high TR and ACR may have been related to methemoglobin formation and/or haemolytic anemia (See supplement Fig. S9 for a putative AOP scheme). These compounds revealed a median TR and ACR of 67 and 42, respectively. Already van Leeuwen et al. (1990b) showed that the toxicity of chloroanilines was increased after 7 days of exposure in the FELS test in contrast to other presumably narcotic compounds such as chlorobenzenes. Metabolic activation was discussed as a potential reason for the increasing toxicity. Some of the aniline derivatives such as diuron or propanil are known to be plant herbicides which interfere with photosynthesis (Koblizek et al., 1998), but this MoA is not relevant for fish toxicity. However, repeated-dose tests conducted for chronic toxicity in mammals indicated that the observed long-term effects might be associated with methemoglobin formation or haemolytic anemia (OECD, 2011). It was shown that aniline derivatives such as propanil are hydrolysed to 3,4-dichloroaniline (3,4-DCA) by acylamidase and that 3,4-DCA or further transformation products can lead to a conversion of 50% of the

hemoglobin to methemoglobin in mice (Chow and Murphy, 1975; McMillan et al., 1990a; McMillan et al., 1990b; McMillan et al., 1991). Similarly, metabolism via 3,4-DCA could be involved in diuron toxicity (Wang et al., 1993). The haemolytic oxidation of erythrocytes by N-hydroxylamines was considered as the reason for hemolytic anemia. Hence, the enhanced toxicity in the FELS might be primarily related to a respiratory distress resulting in a reduced oxygen binding capacity of erythrocytes. Since in embryonic stages the oxygen supply is suggested to be provided mainly by diffusion, this respiratory distress would affect growth and survival only in later life stages (Rombough, 2002; Jacob et al., 2002). Contrary to this hypothesis however, is the higher range of TRs for this MoA when fish embryos are compared to the FELS test. Furthermore, cumulative survival data are typically not reported for the FELS test and/or are not publically available. Therefore, experimental studies targeted to relate respiratory distress to the high ACR and TR of aniline derivatives in fish would be required to confirm the relevance of this mechanism.

We hypothesized that interference with cellular matrix formation represents a further MoA leading to a high median TR (9.3×10^4) and ACR_{AFTS} (89) in the FELS test (see supplement Fig. S9 for putative AOP scheme). This MoA was associated with dithiocarbamate fungicides. The precise fungicidal MoA of dithiocarbamates is not known and they are described to act on multiple sites (Maltby et al., 2009). For mammals, a neuropathic effect via the production of CS_2 was controversially discussed (OPP, 2001). For some but not all dithiocarbamates an inhibition of acetylcholinesterase inhibition was also reported (US EPA, 2001). There are various other MoAs that have been discussed for dithiocarbamates (UN, 2002; OPP, 2001). However, strong evidence that the enhanced toxicity in the FELS test is related to developmental toxicity and inhibition of extracellular matrix formation was provided by studies using zebrafish. Haendel et

al. (2004) and Tilton et al. (2006) observed that exposure of fish embryos to various dithiocarbamates elicited distinct notochord distortions at exposure concentrations starting from 0.08 (sodium metam) and 0.02 μM (thiram). Growth inhibitions in the FELS (LOEC 0.013 to 0.15 μM) test for thiram, ziram, maneb and NaDTMC were observed in a range of concentrations close to those causing notochord distortions in zebrafish embryos (Tilton et al., 2006). This notochord distortion appears to be caused by inhibition of the enzyme lysyl oxidase and repression of the enzyme using antisense morpholinos provoked the same phenotype (van Boxtel et al., 2010). The rescue of the wild type phenotype using triazine as an anaesthetic indicates that notochord malformation require muscle contractions to be established (Tilton et al., 2006). It can be assumed that the notochord distortions may affect swimming behaviour and feeding, leading to the observed reduction in survival and growth observed in the FELS test.

The toxicity of neurotoxic compounds in fish and other vertebrates has been associated with respiratory distress (Candole et al., 1953; Bradbury et al., 2008; Russom et al., 2014). Particularly for acetylcholinesterase inhibitors, the AOP has been well described (Russom et al., 2014). It is known that exposure of fish to neurotoxic compounds can lead to reduced blood oxygen levels caused by interference with the cholinergic system and neuromuscular junctions. Diverse mechanisms or a combination of these, such as haemorrhages in the vertebral column, vasoconstriction of gill sphincter muscles, decreased heart rate and/or reduced movement have been discussed to lower the oxygen level which can ultimately lead to the death of the animal (Bradbury et al., 2008). The dependency on oxygen supply for neurotoxicity is further supported by comparison of acute toxicity between fish embryos and later life stages. Mortality in fish embryos exhibits a weak sensitivity for neurotoxic compounds (Klüver et al., 2015; Knöbel et al., 2012). It is likely that the weak sensitivity of embryos to neurotoxic compounds is not

associated with a lack of the compound's target (e.g., binding to AChE inhibition as the molecular initiating event) but a lack of subsequent key events such as the respiratory distress syndrome – similar as described above for the formation of hemoglobin by aniline derivatives. Hence, the lower median ACR_{AFT} of 5.8 and higher median ACR_{ZFET} of 22 obtained for compounds with a neuromuscular mode of action are plausible due to lack of the respiratory failure syndrome in embryonic stages. The relatively low median TR of 22 may be due to a number of neurotoxic compounds with low ACRs. Many of these compounds exhibit high $\log K_{ow}$ and for these compounds the low effect concentrations for baseline toxicity may result in relatively low TRs. TRs well below 1 observed for a number of hydrophobic compounds may also indicate potential artifacts due to experimental limitations such as water solubility. Another potential reason that requires further investigation and may apply also to other groups of compounds could be the interference with biotransformation. Many organophosphates require metabolic activation and some are also rapidly transformed. This could lead to internal, bioavailable concentrations that differ between different life stages and/or exposure durations (e.g., as discussed for malathion, de Bruijn and Hermens, 1993).

Endocrine disrupting chemicals are considered to be of a high concern primarily due to their ability to interfere with reproduction and subsequent population development (Scholz et al., 2013a; Scholz and Mayer, 2008; Ankley et al., 2010). Reproductive endpoints are not covered in the FELS test. However, our data analysis indicated that growth and survival in the FELS test (median TR=478) were affected for compounds known or discussed to interfere with the endocrine system (17-methyltestosterone, ethinylestradiol, genistein, nonylphenol, tributyltin oxide, see supplement table S1 and S9 for further references) and could be observed at concentrations well below concentrations causing acute toxicity. Some of these compounds

(genistein, tributyltin oxide, Akiyama et al., 1987; WHO, 1999) are known to exhibit also other modes of action and hence, the observed higher TRs may not or not exclusively relate to the endocrine mode of action. It is not precisely known how endocrine disrupting compounds interfere with growth and survival in the FELS test. As shown for ethinylestradiol the interference with regulation of the growth hormone/insulin like growth factor in fish could link estrogenic effects to growth leading to high TRs and ACRs (Shved et al., 2008). A high median ACR (53) was only observed for the comparison of fish embryo test LC₅₀s versus FELS toxicity. This may, however, be caused by a lack of availability of LC₅₀s for adult/juvenile fish for compounds such as methyltestosterone and ethinylestradiol.

Conclusions and perspectives for the development of alternatives to the FELS test

The meta-analysis demonstrated that certain MoAs were associated with an enhanced TR and/or ACR in the FELS test. On the basis of the dataset analysed, neurotoxicity, lysyl oxidase inhibition, endocrine disruption and methemoglobin formation/haemolytic anemia were identified as the (putative) major MoAs leading to high TRs and ACRs in the FELS test. This observation provides a basis to use and develop targeted bioassays linked to the AOP concept (Ankley et al., 2010). These assays could be short-term assays using in vitro cellular or other alternative test systems such as fish embryos and would allow identifying the molecular initiation or a key event leading to the adverse effects, i.e., an enhanced FELS toxicity. Furthermore, MoA-specific QSARs beyond baseline toxicity may be used to predict the FELS toxicity. The analysis of additional data sources for FELS effect concentrations such as ECHA or EFSA dossiers and proprietary databases of environmental agencies may provide additional MoAs that may not have been covered in the present data set.

Given that the FELS test includes the embryonic period, exposure could be restricted to the embryonic period, if endpoints related to relevant MoAs and predictive of chronic toxicity could be identified and already measured in embryonic stages. For some of the major MoAs leading to enhanced ACRs or TRs described here, appropriate fish embryo assays have been developed and described; (1) the alterations in embryonic behavior (movements) have been shown to identify neurotoxicity (Klüver et al., 2015), (2) endocrine disrupting effects can already be detected in fish embryos using, e.g., aromatase as a sensitive target gene in a transgenic zebrafish model (Brion et al., 2012). Likewise, thyroid hormone disrupting compounds (Fetter et al., 2015; Thienpont et al., 2011) and androgens (Sébillot et al., 2014) can be identified using fish embryos. (3) The lysyl oxidase inhibiting dithiocarbamates provoked visible notochord distortion (Tilton et al., 2006; van Boxtel et al., 2010) indicating that the observation of malformations may serve as an indicator of potential enhanced FELS toxicity.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data Availability—Raw data are available as supplement files (S1 and S2).

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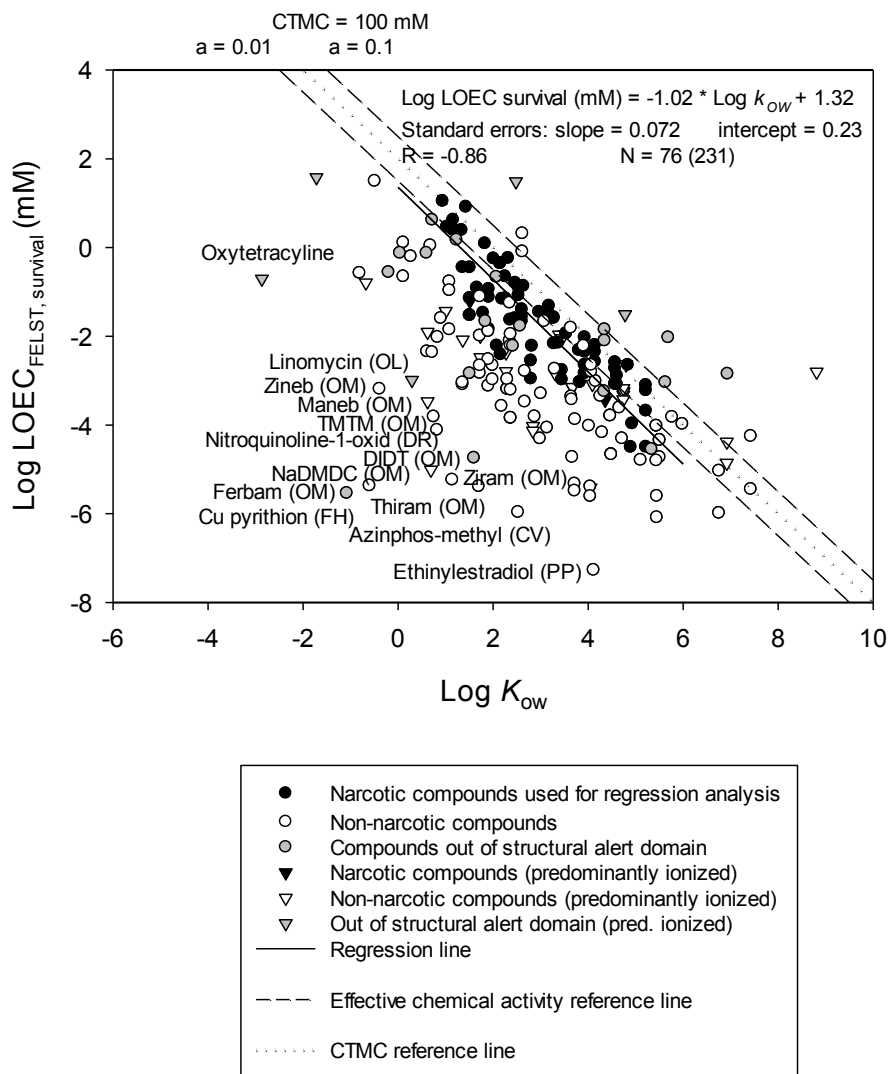
Figure 1. Determination of baseline toxicity for fish early life stage test (FELST) toxicity data (LOEC survival) of different species. Compounds indicated by name exhibited a toxic ratio of more than 1000fold. Ionized chemicals have also been plotted against the log K_{ow} of the neutral form although in reality the actual hydrophobicity will be shifted to the left of the plot depending on the acidity constant pK_a . NaDMDC – sodium dimethyldithiocarbamate, TMTM - tetramethylthiurammonosulfide. “Out of structural alert domain” refers to compounds for which the structures have not been included in the training set for fish acute toxicity structural alerts. *Pimephales promelas* (PP), *Oncorhynchus mykiss* (OM), *Cyprinodon variegatus* (CV), *Danio rerio* (DR) and *Oryzias latipes* (OL). For better visualisation the reference lines for chemical activity and the CTMC (constant toxic membrane concentration) have been drawn over the entire area of the plot which does not represent the valid range (for details on the calculation refer to Material and methods).

Figure 2. Distribution of toxic ratios for different modes of action in the FELS test (details on compound identity, hydrophobicity, LOEC values and the corresponding mode of action can be found in supplement Table S1). The boxes represent the median and the 25 and 75 % percentile. Whiskers represent 1.5 times of the interquartile range and the dots refer to any data outside of the interquartile range. The numbers (n) indicate the number of studies. Some compounds could be represented multiple times. Groups were sorted according to the median TR (toxic ratio). “Out of structural alert domain” refers to compounds of which the structures have not been included in the training set for fish acute toxicity structural alert. Surfactants (n = 3) were excluded from the analysis. “Inh. extracellular matrix” refers to the inhibition of extracellular matrix formation by lysyl oxidase inhibition. Ox. = Oxidative.

Figure 3. Correlation of acute fish toxicity and fish early life stage (FELS) toxicity. Three different endpoints were used for the acute fish toxicity comparison: embryo toxicity in the FELS test (A), acute 96-h fish toxicity (juvenile/adult fish) (B) and zebrafish embryo test (C). For A individual data pairs were derived from the same experiment, for B all data pairs referred to the same species. The indicated sample numbers (n) refer to the number of studies used for regression analysis (in parenthesis the total number of studies is given). For details on compounds and data sources refer to supplemental tables S1 and S6. Indicated compounds represent outliers from the regression analysis (A, B) or compounds with an $ACR_{ZFET} > 45$ (C). The table summarises the parameters of the linear regressions shown in Fig. A,B, and C. CV = *Cyprinodon variegatus*, DR = *Danio rerio*, JF = *Jordanella floridae*, OL = *Oryzias latipes*, OM = *Oncorhynchus mykiss*, PP = *Pimephales promelas*.

Figure 4. Relation of the acute chronic ratio to the mode of action. ACRs were calculated using acute 96-h fish toxicity LC_{50} (A) or acute zebrafish embryo toxicity ($LC_{50, ZFET}$) as references. The dashed line represents an ACR of 10. For details on the compounds and data sources, refer to supplement tables S1, S2 and S6. “Inh. Extracellular matrix” refers to the inhibition of extracellular matrix formation by lysyl oxidase inhibition. Ox. = Oxidative.

Graphical Abstract. Modes of action in the Fish Early Life Stage test that lead to median toxic ratios and acute-to-chronic ratios ($ACR \geq 10$). The ACR was calculated using acute toxicity in juvenile/adult fish (AFT) or zebrafish embryos (ZFET) as reference values.

**Fig. 1**

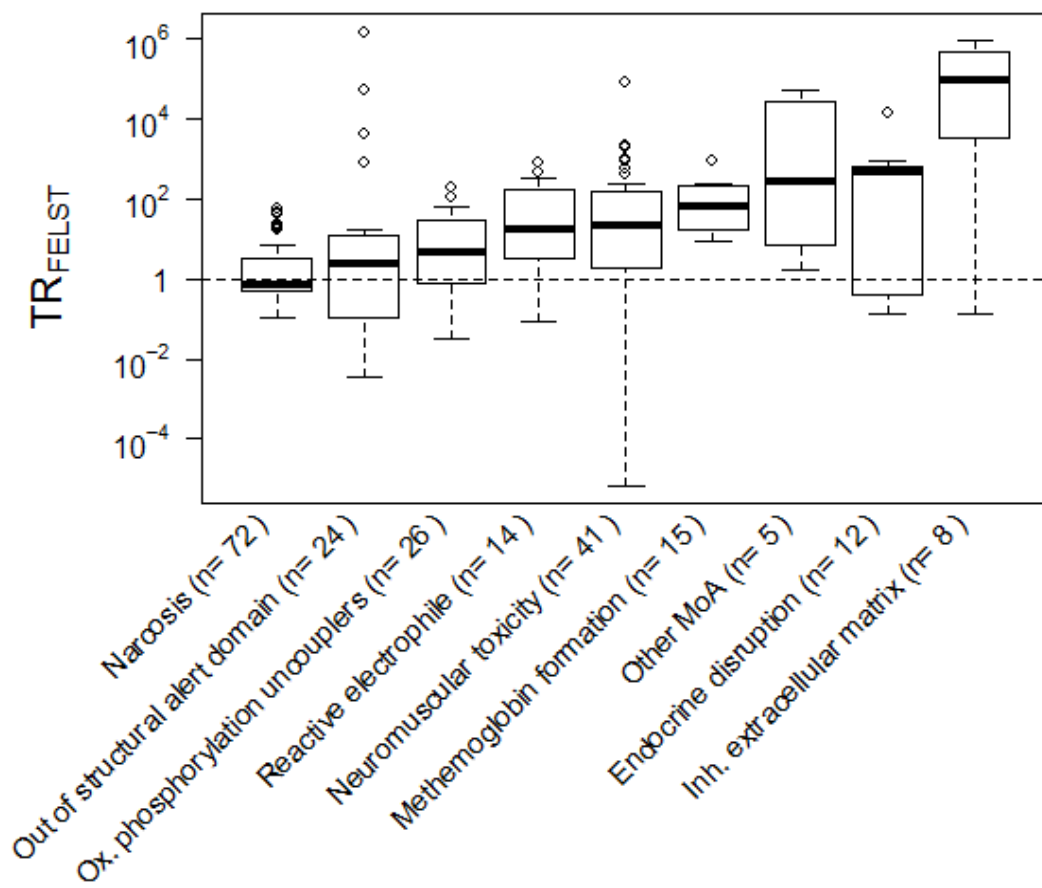


Fig. 2

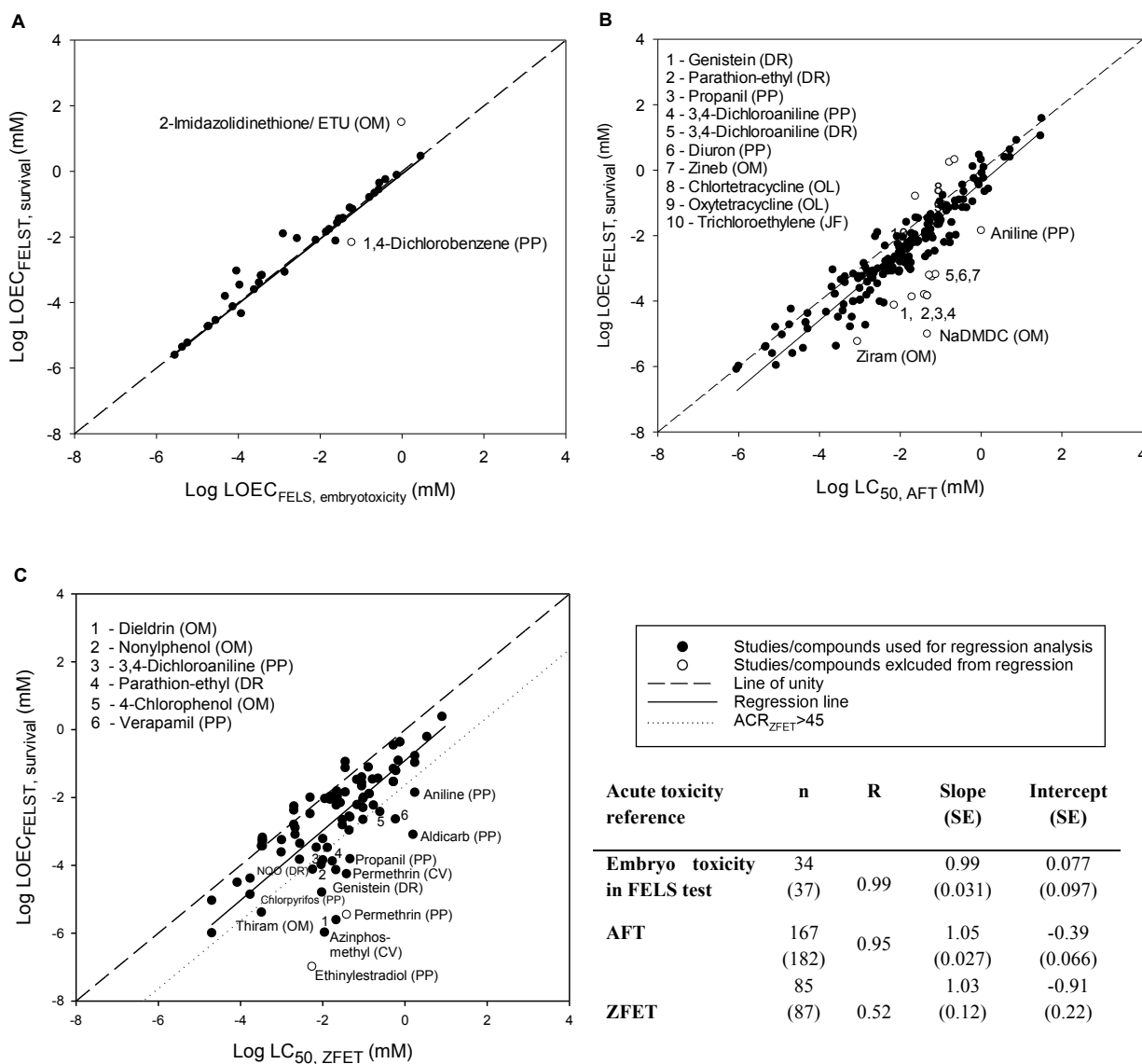
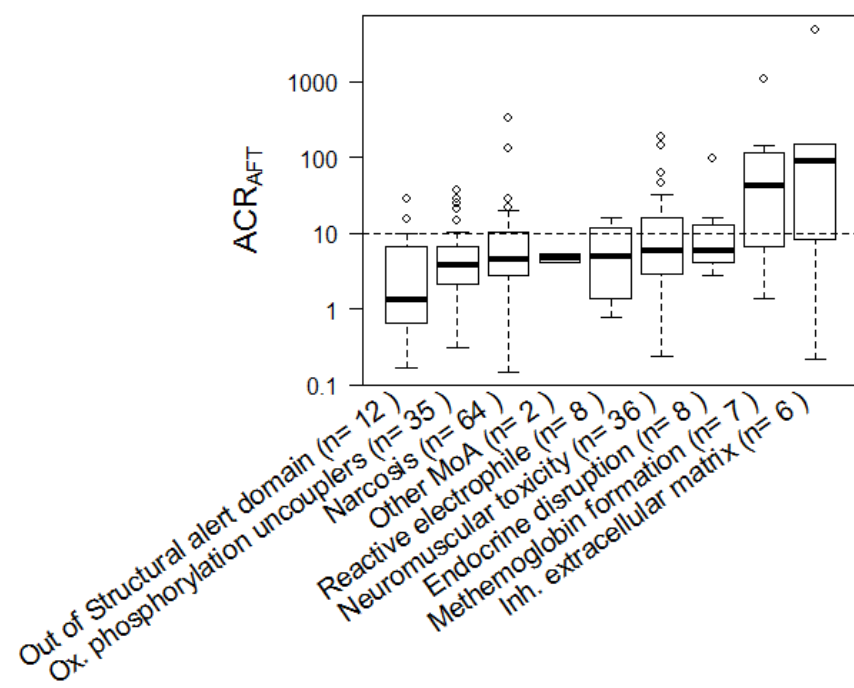
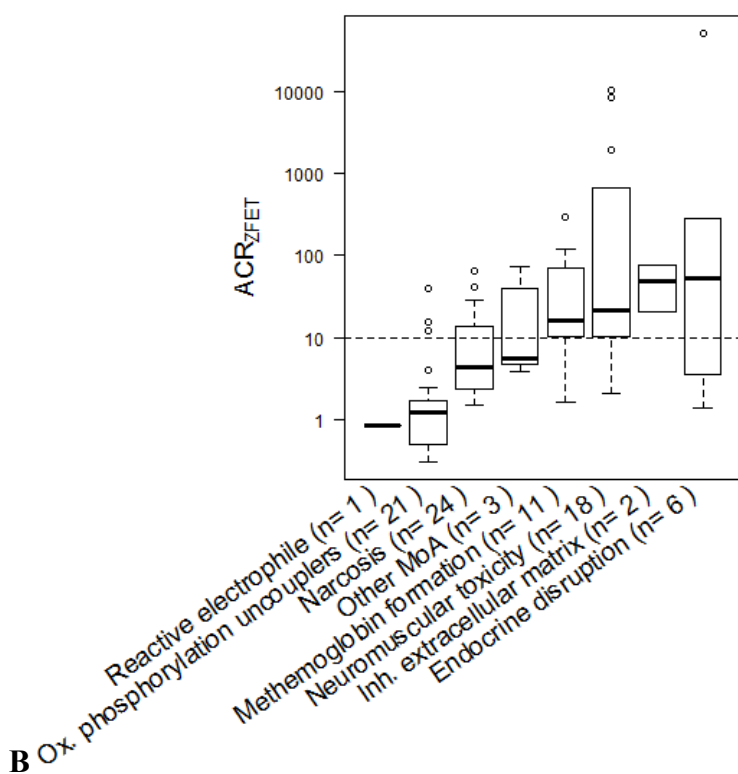


Fig. 3

A**B****Fig. 4**